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(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BROEKAERT, Willem, Frans [BE/BE]; Kluizenbosstraat 26, B-1700 Dilbeck (BE). DE SAMBLANX, Genovera, Wivina [BE/BE]; Willem de Croylaan 42, B-3001 Heverlee (BE). REES, Sarah, Bronwen [GB/GB]; 32 Micheldever Way, Forest Park, Bracknell, Berkshire RG12 3XX (GB).

(74) Agents: HUSKISSON, Frank, Mackie et al.; ZENECA Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).

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(57) Abstract

Antifungal proteins which are analogues of the Rs-AFP2 protein and contain particular mutations in their amino acid sequence. The mutated proteins possess enhanced salt-tolerant antifungal activity. The proteins are useful for combating fungal diseases in agricultural, pharmaceutical or preservative applications.

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ANTIFUNGAL PROTEINS

This invention relates to antifungal proteins, processes for their manufacture and use, and DNA sequences encoding them.

In this context, antifungal proteins are defined as proteins or peptides possessing antifungal activity. Activity includes a range of antagonistic effects such as partial inhibition or death.

A wide range of antifungal proteins with activity against plant pathogenic fungi have been isolated from certain plant species. We have previously described a class of antifungal proteins capable of isolation from radish and other plant species. These proteins are described in the following publications which are specifically incorporated herein by reference: International Patent Application Publication Number WO93/05153 published 18 March 1993; Terras FRG et al, 1992, J Biol Chem, 267:15301-15309; Terras et al, FEBS Lett, 1993, 316:233-240; Terras et al, 1995, Plant Cell, 7:573-588. The class includes Rs-AFP1 (antifungal protein 1), Rs-AFP2, Rs-AFP3 and Rs-AFP4 from Raphanus sativus and homologous proteins such as Bn-AFP1 and Bn-AFP2 from Brassica napus, Br-AFP1 and Br-AFP2 from Brassica rapa, Sa-AFP1 and Sa-AFP2 from Sinapis alba, At-AFP1 from Arabidopsis thaliana, Dm-AMP1 and Dm-AMP2 from Dahlia merckii, Cb-AMP1 and Cb-AMP2 from Cnicus benedictus, Lc-AFP from Lathyrus cicera, Ct-AMP1 and Ct-AMP2 from Clitoria ternatea. The proteins specifically inhibit a range of fungi and may be used as fungicides for agricultural or pharmaceutical or preservative purposes. It has been proposed that this class of antifungal proteins should be named as plant defensins (Terras F.R.G. et al 1995, Plant Cell 7 573-588) and these proteins share a similar motif of conserved cysteines and glycines (Broekaert et al 1995 Plant Physiol 108 1353-1358).

Figure 1 shows the amino acid sequences of the protein Rs-AFP2 and the substantially homologous proteins Rs-AFP1, Rs-AFP3, Rs-AFP4, Br-AFP1, Br-AFP2, Bn-AFP1, Bn-AFP2, Sa-AFP1, Sa-AFP2 and At-AFP1 which are small 5kDa polypeptides that are highly basic and rich in cysteine. Figure 1 numbers the positions of the amino acid residues: the dash (-) at the start of the Rs-AFP3 sequence indicates a gap introduced for maximum alignment. The sequences shown

for Br-AFP1, Br-AFP2, Bn-AFP1, Bn-AFP2, Sa-AFP1, Sa-AFP2 and At-AFP1 are not complete: only the N-terminal sequences are shown. The question mark (?) in the Bn-AFP2 sequence indicates a non-standard amino acid which the sequencing could not assign and which is thought to be a post-translational modification on one of the standard amino acid residues.

The primary structures of the two antifungal protein isoforms capable of isolation from radish seeds, Rs-AFP1 and Rs-AFP2, only differ at two positions: the glutamic acid residue (E) at position 5 in Rs-AFP1 is a glutamine residue (Q) in Rs-AFP2, and the asparagine residue (N) at position 27 in Rs-AFP1 is substituted by an arginine residue (R) in Rs-AFP2. As a result, Rs-AFP2 has a higher net positive charge (+2) at physiological pH. Although both Rs-AFPs are 94% identical at the amino acid sequence level, Rs-AFP2 is two- to thirty-fold more active than Rs-AFP1 on various fungi and shows an increased salt-tolerence. The proteins Rs-AFP3 and Rs-AFP4 are found in radish leaves following localized fungal infection. The induced leaf proteins are homologous to Rs-AFP1 and Rs-AFP2 and exert similar antifungal activity in vitro.

The cDNA encoding Rs-AFP1 encodes a preprotein with a signal peptide followed by the mature protein. The cDNA sequence is shown in Figure 2. Saccharomyces cerevisiae can be used as a vector for the production and secretion of Rs-AFP2 (Vilas Alves et al, FEBS Lett, 1994, 348:228-232). Plant-derivable "wild-type" Rs-AFP2 can be correctly processed and secreted by yeast when expressed as a N-terminal fusion to the yeast mating factor α1 (MFα1) preprosequence. The Rs-AFP2 protein does not have adverse effects on yeast even at concentrations as high as 500 μg/ml.

We now provide new potent antifungal proteins based on the structure of the Rs-AFPs and related proteins.

According to a first aspect the invention provides an antifungal protein having an amino acid sequence which is substantially homologous to the Rs-AFP2 sequence shown in Figure 1 and containing at least one mutation selected from the group consisting of a basic residue at position 9, a basic residue at position 39, a hydrophobic residue at position 5 and a hydrophobic residue at position 16.

According to a preferred embodiment of the first aspect of the present invention there is provided an antifungal protein having an amino acid sequence which is substantially homologous to the Rs-AFP2 sequence shown in Figure 1 and containing at least one mutation selected from the group consisting of an arginine residue at position 9, an arginine residue at position 39, a methionine residue at position 5 and a methionine residue at position 16. An antifungal protein having both a mutation to arginine at position 9 and a mutation to arginine at position 39 may be particularly active.

Proteins which are substantially homologous to the Rs-AFP2 protein include the proteins Rs-AFP1, Rs-AFP3, Rs-AFP4, Br-AFP1, Br-AFP2, Bn-AFP1, Bn-AFP2, Sa-AFP1, Sa-AFP2 and At-AFP1 shown in Figure 1.

As used herein the term substantially homologous denotes those proteins which have an amino acid sequence with at least 40% identity, preferably at least 60% identity and most preferably at least 80% identity to the Rs-AFP2 sequence.

The invention further provides an antifungal peptide which comprises at least six amino acid residues identical to a run of amino acid residues in an antifungal protein according to the invention, said run of residues including at least one of the mutated residues.

In particular, there are provided the following antifungal proteins and antifungal peptides derived therefrom:

a protein having the amino acid sequence of Rs-AFP1, Rs-AFP2, Rs-AFP3 or Rs-AFP4 in which the glycine residue at postion 9 is replaced by an arginine residue;

a protein having the amino acid sequence of Rs-AFP1, Rs-AFP2 or Rs-AFP3 in which the valine residue at postion 39 is replaced by an arginine residue;

a protein having the amino acid sequence of Rs-AFP4 in which the isoleucine residue at postion 39 is replaced by an arginine residue;

a protein having the amino acid sequence of Rs-AFP1, Rs-AFP2 or Rs-AFP3 in which the glycine residue at postion 9 is replaced by an arginine residue and the valine residue at position 39 is replaced by an arginine residue;

a protein having the amino acid sequence of Rs-AFP4 in which the glycine residue at postion 9 is replaced by an arginine residue and the isoleucine residue at position 39 is replaced by an arginine residue;

a protein having the amino acid sequence of Rs-AFP1, Rs-AFP3 or Rs-AFP4 in which the glutamic acid residue at postion 5 is replaced by a methionine residue;

a protein having the amino acid sequence of Rs-AFP2 in which the glutamine residue at postion 5 is replaced by a methionine residue;

a protein having the amino acid sequence of Rs-AFP1, RS-AFP2, Rs-AFP3 or Rs-AFP4 in which the glycine residue at postion 16 is replaced by a methionine residue.

Proteins according to the invention include proteins having one of the following sequences:

QKLCERPSRTWSGVCGNNNACKNQCINLEKARHGSCNYVFPAHKCICYFPC;

QKLCERPSGTWSGVCGNNNACKNQCINLEKARHGSCNYRFPAHKCICYFPC;

QKLCERPSRTWSGVCGNNNACKNQCINLEKARHGSCNYRFPAHKCICYFPC;

QKLCMRPSGTWSGVCGNNNACKNQCINLEKARHGSCNYVFPAHKCICYFPC;

QKLCERPSGTWSGVCMNNNACKNQCINLEKARHGSCNYVFPAHKCICYFPC;

QKLCQRPSRTWSGVCGNNNACKNQCIRLEKARHGSCNYVFPAHKCICYFPC;

QKLCQRPSGTWSGVCGNNNACKNQCIRLEKARHGSCNYRFPAHKCICYFPC;

QKLCQRPSRTWSGVCGNNNACKNQCIRLEKARHGSCNYRFPAHKCICYFPC;

QKLCMRPSGTWSGVCGNNNACKNQCIRLEKARHGSCNYVFPAHKCICYFPC;

QKLCQRPSGTWSGVCMNNNACKNQCIRLEKARHGSCNYVFPAHKCICYFPC;

KLCERSSRTWSGVCGNNNACKNQCIRLEGAQHGSCNYVFPAHKCICYFPC;

KLCERSSGTWSGVCGNNNACKNQCIRLEGAQHGSCNYRFPAHKCICYFPC;

KLCERSSRTWSGVCGNNNACKNQCIRLEGAQHGSCNYRFPAHKCICYFPC;

KLCMRSSGTWSGVCGNNNACKNQCIRLEGAQHGSCNYVFPAHKCICYFPC;

KLCERSSGTWSGVCMNNNACKNQCIRLEGAQHGSCNYVFPAHKCICYFPC;

QKLCERSSRTWSGVCGNNNACKNQCINLEGARHGSCNYIFPYHRCICYFPC;

QKLCERSSGTWSGVCGNNNACKNQCINLEGARHGSCNYRFPYHRCICYFPC;

QKLCERSSRTWSGVCGNNNACKNQCINLEGARHGSCNYRFPYHRCICYFPC;

QKLCMRSSGTWSGVCGNNNACKNQCINLEGARHGSCNYIFPYHRCICYFPC;

QKLCERSSGTWSGVCMNNNACKNQCINLEGARHGSCNYIFPYHRCICYFPC.

A cDNA clone encoding the plant-derivable "wild-type" Rs-AFP2 preprotein was modified by recombinant DNA methods in order to allow expression in the yeast Saccharomyces cerevisiae. This peptide was expressed in yeast as a fusion protein carrying at its N-terminus the prepro sequences derived from the precursor of the yeast pheromone mating factor α1. These sequences allow secretion of the biologically active peptide in a correctly processed form. The yeast expression system was then used to express and characterize isoforms of the Rs-AFP2 protein by introducing deliberate or random changes into the coding region. These isoforms were subsequently purified and tested for their antifungal activity.

The Rs-AFP2 isoform having a mutation at position 5 (glutamine to methionine) and the Rs-AFP2 isoform having a mutation at position 16 (glycine to methionine) have an enhanced salt-tolerant antifungal activity. However, two other isoforms were found to possess particularly advantageous antifungal properties. The Rs-AFP2 isoform having a mutation at position 9 (glycine to arginine) and the Rs-AFP2 isoform having a mutation at position 39 (valine to arginine) have a significantly enhanced antifungal activity. This enhanced activity is prominent in high salt conditions. An Rs-AFP2 isoform having a mutation at both position 9 (glycine to arginine) and at position 39 (valine to arginine) may have an even greater salt-tolerance.

Proteins which maintain their antifungal activity as salt concentration is increased are particularly suitable for use as antifungal agents in higher salt conditions. For example, such proteins are particularly suitable for expression within some biological organisms including plants. The most abundant divalent cations in plant tissues are Ca^{2+} and Mg^{2+}. The concentration of free Ca^{2+} in the cytosol is very low (0.1 to 1 μM) (Macklom, 1984, Plant Cell Environ, 7:407-413)), whereas free Mg^{2+} reaches about 1 mM (Hepler and Wyne, 1982, Ann Rev Plant Physiol, 36:397-439). Free Ca^{2+} in plant vacuoles is about 0.06 to 1 mM and apoplastic free Ca^{2+} ranges between 0.02 and 1.3 mM (Harker and Venis, 1991, Plant Cell Environ, 14:525-530). It thus appears that relatively high ionic strength conditions occur in

all cellular compartments. In many cases, however, fungal infection leads to the disruption of the cells and contact of the cellular contents with the environment. Therefore it is difficult to predict the exact ionic conditions under which antifungal proteins expressed within a plant cell will interact with invading hyphae. However, proteins whose antifungal activity is less sensitive to cation concentration are particularly suitable for expression within plant cells.

An antifungal protein according to the invention may be manufactured from its known amino acid sequence by chemical synthesis using a standard peptide synthesiser, or produced within a suitable organism (for example, a micro-organism or plant) by expression of recombinant DNA. The antifungal protein is useful as a fungicide and may be used for agricultural or pharmaceutical applications.

Knowledge of its primary structure enables manufacture of the antifungal protein, or parts thereof, by chemical synthesis using a standard peptide synthesiser. It also enables production of DNA constructs encoding the antifungal protein.

The invention further provides a DNA sequence encoding an antifungal protein according to the invention. The DNA sequence may be predicted from the known amino acid sequence and DNA encoding the protein may be manufactured using a standard nucleic acid synthesiser. Alternatively, DNA encoding proteins according to the invention may be produced by appropriate site-directed mutagenesis of DNA sequences encoding one of the proteins shown in Figure 1.

The DNA sequence encoding the antifungal protein may be incorporated into a DNA construct or vector in combination with suitable regulatory sequences (promoter, terminator, transit peptide etc). The DNA sequence may be placed under the control of a homologous or heterologous promoter which may be a constitutive or an inducible promoter (stimulated by, for example, environmental conditions, presence of a pathogen, presence of a chemical). The transit peptide may be a homologous or heterologous to the antifungal protein and will be chosen to ensure secretion to the desired organelle or to the extracellular space. The transit peptide is preferably that naturally associated with the antifungal protein of interest.

Such a DNA construct may be cloned or transformed into a biological system which allows expression of the encoded protein or an active part of the protein.

Suitable biological systems include micro-organisms (for example, bacteria such as Escherichia coli, Pseudomonas and endophytes such as Clavibacter xyli subsp. cynodontis (Cxc); yeast; viruses; bacteriophages; etc), cultured cells (such as insect cells, mammalian cells) and plants. In some cases, the expressed protein may subsequently be extracted and isolated for use.

An antifungal protein according to the invention is useful for combatting fungal diseases in plants. The invention further provides a process of combating fungi whereby they are exposed to an antifungal protein according to the invention.

For pharmaceutical applications, the antifungal protein may be used as a fungicide to treat mammalian infections (for example, to combat yeasts such as Candida).

An antifungal protein according to the invention may also be used as a preservative (for example, as a food additive).

For agricultural applications, the antifungal protein may be used to improve the disease-resistance or disease-tolerance of crops either during the life of the plant or for post-harvest crop protection. Pathogens exposed to the proteins are inhibited. The antifungal protein may eradicate a pathogen already established on the plant or may protect the plant from future pathogen attack. The eradicant effect of the protein is particularly advantageous.

Exposure of a plant pathogen to an antifungal protein may be achieved in various ways, for example:

(a) The isolated protein may be applied to plant parts or to the soil or other growth medium surrounding the roots of the plants or to the seed of the plant before it is sown using standard agricultural techniques (such as spraying).

The protein may have been extracted from plant tissue or chemically synthesised or extracted from micro-organisms genetically modified to express the protein. The protein may be applied to plants or to the plant growth medium in the form of a composition comprising the protein in admixture with a solid or liquid diluent and optionally various adjuvants such as surface-active agents. Solid compositions may be in the form of dispersible powders, granules, or grains.

(b) A composition comprising a micro-organism genetically modified to express the antifungal protein may be applied to a plant or the soil in which a plant grows.

(c) An endophyte genetically modified to express the antifungal protein may be introduced into the plant tissue (for example, via a seed treatment process).

An endophyte is defined as a micro-organism having the ability to enter into non-pathogenic endosymbiotic relationships with a plant host. A method of endophyte-enhanced protection of plants has been described in a series of patent applications by Crop Genetics International Corporation (for example, International Application Publication Number WO90/13224, European Patent Publication Number EP-125468-B1, International Application Publication Number WO91/10363, International Application Publication Number WO87/03303). The endophyte may be genetically modified to produce agricultural chemicals. International Patent Application Publication Number WO94/16076 (ZENECA Limited) describes the use of endophytes which have been genetically modified to express a plant-derived antifungal protein.

(d) DNA encoding an antifungal protein may be introduced into the plant genome so that the protein is expressed within the plant body (the DNA may be cDNA, genomic DNA or DNA manufactured using a standard nucleic acid synthesiser).

Plant cells may be transformed with recombinant DNA constructs according to a variety of known methods (Agrobacterium Ti plasmids, electroporation, microinjection, microprojectile gun, etc). The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocotyledonous and dicotyledonous plants may be obtained in this way, although the latter are usually more easy to regenerate. Some of the progeny of these primary transformants will inherit the recombinant DNA encoding the antifungal protein(s).

The invention further provides a plant having improved resistance to a fungal pathogen and containing recombinant DNA which expresses an antifungal protein

according to the invention. Such a plant may be used as a parent in standard plant breeding crosses to develop hybrids and lines having improved fungal resistance.

Recombinant DNA is DNA , preferably heterologous, which has been introduced into the plant or its ancestors by transformation. The recombinant DNA encodes an antifungal protein expressed for delivery to a site of pathogen attack (such as the leaves). The DNA may encode an active subunit of an antifungal protein.

A pathogen may be any fungus growing on, in or near the plant. In this context, improved resistance is defined as enhanced tolerance to a fungal pathogen when compared to a wild-type plant. Resistance may vary from a slight increase in tolerance to the effects of the pathogen (where the pathogen in partially inhibited) to total resistance so that the plant is unaffected by the presence of pathogen (where the pathogen is severely inhibited or killed). An increased level of resistance against a particular pathogen or resistance against a wider spectrum of pathogens may both constitute an improvement in resistance. Transgenic plants (or plants derived therefrom) showing improved resistance are selected following plant transformation or subsequent crossing.

Where the antifungal protein is expressed within a transgenic plant or its progeny, the fungus is exposed to the protein at the site of pathogen attack on the plant. In particular, by use of appropriate gene regulatory sequences, the protein may be produced in vivo when and where it will be most effective. For example, the protein may be produced within parts of the plant where it is not normally expressed in quantity but where disease resistance is important (such as in the leaves).

Examples of genetically modified plants which may be produced include field crops, cereals, fruit and vegetables such as: canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

The invention will now be described by way of example only, with reference to the following drawings wherein:

Figure 1 shows the amino acid sequences of the Rs-AFPs and related proteins.

Figure 2 shows the nucleotide sequence of the cDNA encoding Rs-AFP1.

Figure 3 shows the schematic representation of the construction of the expression vectors pMFprepro/RsAFP2 and pMFpre/RsAFP2.

Figure 4 shows the amino acid sequences of plant-derivable Rs-AFP2, and a series of yeast-expressed Rs-AFP2 (yRs-AFP2) isoforms.

Figure 5 illustrates PCR amplification using the mutagenic primer OWB41 and the M13 reverse primer.

Figure 6 is a graph of relative specific antifungal activity ($1/IC_{50}$) on F culmorum of the Rs-AFP isoforms.

Figure 7 is a graph of the percentage growth inhibition of F culmorum caused by Rs-AFP2 isoforms in varying concentrations of $CaCl_2$ (panel A) and KCl (panel B).

EXAMPLE 1

Construction of Expression Vectors for Secretion of Rs-AFP2 by Yeast

Saccharomyces cerevisiae can be used as a vector for the production and secretion of Rs-AFP2 as described by Vilas Alves et al, FEBS Lett, 1994, 348:228-232 using the method described below.

Plasmid pFRG1 is a pBluescript IISK derivative containing a full length cDNA clone encoding Rs-AFP1 (international patent application publication number WO93/05153). By PCR-mediated site-directed mutagenesis (Merino et al, 1992, BioTechniques, 12:508-510) two mutations were introduced such that the encoded protein is the more active isoform Rs-AFP2. A third mutation (CGA to CGT for Arg^{31} of mature Rs-AFP2) was introduced to comply with the codon usage preference in Saccharomyses cerevisiae (Bennetzen and Hall, 1982, J Biol Chem, 257:3026). The resulting plasmid was called pBluescript/RsAFP2.

The vectors pMFpre/RsAFP2 and pMFprepro/RsAFP2 are based on the yeast/E coli shuttle vector pTG3828 (Achstetter et al, 1992, Gene, 110:25-31). pTG3828 contains a URA3-d selection marker, the origin of replication from the

yeast 2 μ plasmid, the prokaryotic ColE1 origin of replication and the ampicillin resistance marker from pBR322. pTG3828 also contains the yeast phosphoglycerate kinase (PGK) terminator preceded by a polylinker with multiple unique restriction sites which facilitate insertion of an expression block.

The expression blocks in pMFpre/RsAFP2 and pMF prepro/RsAFP2 were derived from the M13 phage derivative M13TG5879 (Reichhart et al, 1992, Invertebrate Reproduction and Development, 21:15-24) which contains the promoter of the yeast MFα1 gene, the coding region of the MFα1 pre-sequence with an engineered NheI site, and the coding region of the MFα1 pro-sequence with an engineered HindIII site. The expression cassette of M13TG5879 was amplified by PCR using the sense primer OWB63:
5'TATCAGTCGACGCATGCTATTGATAAGATTTAAAGG (SalI site underlined, SphI site in bold), which introduces a novel SalI site immediately adjacent to the SphI site at the 5' end of the MFαl promoter, and the M13 reverse primer as an antisense primer. The resulting PCR product was digested with SalI-BamHI and subcloned into pBluescriptII SK to yield pVD4.

Plasmid pBluescript/RsAFP2 was used as a template for the amplification of the coding sequence of mature Rs-AFP2 in two separate PCR reactions. In the first PCR reaction the sense primer OWB61:
5'AATAAGCTTGGACAAGAGACAGAAGTTGTGCCAAAGG (HindIII site underlined) was designed such that sixteen extra nucleotides (coding for the last five amino acids of the MFαl pro-sequence) were added upstream of the coding region of mature Rs-AFP2. The HindIII site allowed in frame cloning into the HindIII site in the MFαl prosequence region of pVD4 Reichhart JM et al, 1991, Invertebrate Reproduction and Development 21:15-24) . The antisense primer OWB64:
5'AAGGATCCCTATTAACAAGGAAAGTAGC (BamHI site underlined) introduced a second stop codon and a BamHI site immediately downstream of the stop codon of the coding region of Rs-AFP2. In the second PCR reaction, the same antisense primer was combined with the sense primer OWB62:
5'AATGCTAGCTCAGAAGTTGTGCCAAAGG (NheI site underlined) which added seven extra nucleotides (coding for the last two amino acids of the MFα1

presequence), including a NheI site (for in frame cloning into the NheI site in the MF αl presequence region of pVD4) upstream of the coding region of mature Rs-AFP2. The fragments corresponding to the mature domain of Rs-AFP2 obtained by PCR amplification in the first or in the second reaction were digested with HindIII-BamH1 and NheI-BamHI, respectively, and introduced in the corresponding sites of pVD4 to yield vectors pVD5 and pVD6, respectively. The resulting vectors were digested with SalI-BamHI to isolate the expression blocks, which were then subcloned into SalI-BglII digested pTG3828 to yield the vectors pMFpre-RsAFP2 and pMFprepro/RSAFP2, respectively.

Figure 3 shows the schematic representation of the construction of the expression vectors pMFprepro/RsAFP2 and pMFpre/RsAFP2. The different steps in the procedure are (1) PCR amplification of the coding region of mature RsAFP2 using primers to add a HindIII site and part of the MFα1 pro region (5' site) and a BamHI site (3' site); (2) PCR amplification of the coding region of mature RsAFP2 using primers to introduce a NheI and part of the MFα1 pre region (5' site) and a BamHI (3' site); (3) subcloning of the PCR product into HindIII-BamHI digested pVD4; (4) subcloning of the PCR product into NheI-BamHI digested pVD4; (5) digestion of the resulting plasmids with SalI/BamHI and subcloning of the inserts in SalI-Bg1II digested pTG3828. (Abbreviations in Figure 3: pre, signal sequence domain of RsAFP1 cDNA; preα, signal sequence domain of MFα1 gene; proα, propeptide domain of MFα1 gene; pMFα 1, promoter domain of MFα1 gene; tPGK, terminator domain of the yeast phosphoglycerate kinase gene).

The plasmids pMFpre-RsAFP2, pMFprepro/RSAFP2 and pTG3828 were transformed in yeast (S cerevisiae) strain cl3-ABYS86 (genotype; α pra1-1, prb1-1, prc1-1, cps1-3, ura3-5, leu2-3, 112, his-) by the lithium acetate method as described by Elble (1992, BioTechniques, 13:18). Selection of transformants was done on minimal selective SD medium lacking uracil (Sherman, 1991, Meth Enzymol, 194:3-21). Presence of the plasmids in the yeast colonies was verified by PCR as described by Ward (1990, Nucl Acids Res, 18:5319).

EXAMPLE 2

Purification and Analysis of Yeast-Expressed Rs-AFP2

Yeast cells transformed with either pTG3828, pMFprepro/Rs AFP2 or PMFpre/RsAFP2 were grown in selective SD medium until a saturated culture was obtained. To assess the antifungal activity of proteins secreted by the yeast cells, the supernatant of the yeast cultures was filtered (sterile 0.22 µm filter) and serially diluted in sterile water. Diluted sampels (20 µl) were incubated in microtiter plate wells with 80 µl of half strength potato dextrose broth (Difco) containing spores 10^4 spores/ml) of Fusarium culmorum.

Growth of the fungi was monitored by microspectrophotometry as described by Broekaert et al (1990, FEMS Microbiol Lett, 69:55-60). Homogenates of yeast cells were prepared by spinning down 1 ml of a saturated yeast culture, suspending the cells in 200 µl of water, vortexing the cells in the presence of 0.2g of glass beads (425-600 µm), and clearing the homogenate by centrifugation (1 min, 10000xg). Antifungal activity could only be detected in the culture medium of yeast cells transformed with pMFprepro/RsAFP2, which contained about 2 µg/ml of Rs-AFP2 equivalents. The activity of the homogenate of these cells, as well as that of culture media and cell homogenates of yeast cells transformed with pMFpre/RsAFP2 or pTG3828 transformed yeasts was below the detection limit (about 0.2 µg/ml of Rs-AFP2 equivalents). Hence, pMFprepro/RsAFP2 seems to convey significant expression of Rs-AFP2 in yeast.

The supernatant of 100 ml of a saturated culture of yeast transformed with pMFprepro/RsAFP2 (grown on minimal selective SD medium supplemented with 0.5 % w/v of casamino acids) was centrifuged (4000 rpm, 10 min), and filtered (0.45 µm) to remove yeast cells and debris. Tris-HCl (pH9) was added to the supernatant to a final concentration of 50 mM. The sample was loaded at a flow rate of 2 ml/min on an anion exchange chromatography column (Q-Sepharose Fast Flow, 20 ml bed volume, Pharmacia), on-line connected with a disposable reversed-phase C8 silica column (Bond Elut, 500 mg solid phase, Varian, Harbor City, USA). The antifungal activity was not retained on the Q-Sepharose matrix but bound to the C8 silica matrix. The C8 silica column was rinsed with 6 ml of 10% (v/v) acetonitrile in 0.1% (v/v) trifluroacetic acid (TFA) and subsequently eluted with 4 ml of 30% (v/v) acetonitrile in 0.1% (v/v) TFA. The latter eluate was dried in a rotating vacuum

concentrator, redissolved in 0.5 ml 15% (v/v) acetonitrile containing 0.1 % (v/v) TFA, and was loaded on a reversed-phase C2/C18 silica column (Pep-S, 5 μm beads, 0.4 x 25 cm, Pharmacia connected to a Waters 600 HPLC station pre-equilibriated with 15% acetonitrile containing 0.1% (v/v) TFA. After loading, the column was rinsed with the same buffer until the absorbance reached background level. The column was subsequently eluted with a 15 minute linear gradient from 15% to 50% acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min. The eluate was monitored for proteins by on-line measurement of the absorbance at 280 nm. Peak fractions were collected manually, dried in a rotating vacuum concentrator to remove the solvents, and redissolved in 200 μl of distilled water. Twenty μl fractions were tested in a liquid growth inhibition assay: 20 μl samples were incubated in microtiter plate wells with 80 μl of half strength potato dextrose broth (Difco) containing 10^4 spores/ml of F culmorum; growth of the fungi was monitored by microspectrophotometry as described by Broekaert et al (1990, FEMS Microbiol Lett, 69: 55-60).

Only the main peak (peak A, elution time 14.7 min) and a smaller peak (peak B, elution time 15.2 min) coeluted with antifungal activity. The elution time of peak B was identical to that of plant-derivable Rs-AFP2 (15.2 min).

The amino-terminal amino acid sequence obtained by automated Edman degradation for RPC-purified peak A revealed a sequence of 51 amino acids, all of which being identical to the sequence of Rs-AFP2. This sequence includes an N-terminal glutamine which is known to be blocked by cyclisation in plant-derivable Rs-AFP2 (Terras et al, 1992, J Biol Chem, 267:15301-15309). Absence of any contaminating signals in the amino acid sequence analysis indicated that the peak A fraction was essentially homogeneous. No sequence signals could be recorded for RPC-purified peak B material, probably due to blocking of its N-terminus. This protein fraction was treated with pyroglutamate aminopeptidase in order to cleave off the presumed blocked glutamine residue, but also in this case no amino acid sequence could be determined, whereas the same treatment successfully deblocked plant-derivable Rs-AFP2. Because of the uncertain identification of the peak B

material and because of its lower abundancy relative to peak A material, the peak B material was not further analysed.

The specific antifungal activity of RPC-purified peak A material, as well as that of plant-derivable Rs-AFP2, was determined by measuring the percentage growth inhibition of F culmorum caused by serial dilutions of the protein samples. The IC_{50} values (concentration required for 50% growth inhibition) values derived from dose-response curves, was about 3 µg/ml for both protein preparataions. Moreover, the type of inhibition caused by RPC-purified peak A material was identical to that caused by plant-derivable Rs-AFP2, showing a characteristic morphological distortion of the fungal hyphae typified by the induction of multiple branches near the tips.

These results show that yeast cells transformed with pMFprepro/Rs-AFP2 produce a protein that has the same biological activity as plant-derivable Rs-AFP2. Presence of the MFα1 preprosequence seems to be essential for expression of Rs-AFP2 in yeast.

EXAMPLE 3

Production of Rs-AFP2 isoforms containing amino acid mutations

In order to produce Rs-AFP2 isoforms with single amino acid substitutions or deletions, mutations were introduced by PCR-directed mutagenesis in the DNA region coding for the mature Rs-AFP2 domain.

Figure 4 shows the amino acid sequences of plant-derivable wild-type protein (Rs-AFP2), yeast-expressed Rs-AFP2 (yRs-AFP2), Sorghum bicolor α-amylase inhibitor 2 (SIα2) and four series of yeast-expressed isoforms of Rs-AFP2 with single amino acid substitutions or deletions. Z indicates a pyroglutamyl residue. Amino acids identical to the corresponding residue in Rs-AFP2 are indicated by dots whereas amino acid deletions relative to the Rs-AFP2 sequence are represented by a dash.

The yRS-AFP2 isoforms in Series A (Figure 4) include a range of mutations in Rs-AFP2, some of which represent a substitution by the corresponding amino acid occurring in SIα2 (Bloch and Richardson, 1991, FEBS Lett, 279:101-104). SIα2 is a protein which is partially homologous to Rs-AFP2 but which (in contrast to

Rs-AFP2) does not exhibit antifungal activity when assayed as described in Example 2. Series B contains proline deletions. In Series C, particular amino acids were replaced by a basic residue (arginine) in order to obtain more basic Rs-AFP2 isoforms.

The vector for production of yRS-AFP/Q5M, the Rs-AFP2 isoform with an amino acid substitution at position 5 (glutamine to methionine) was prepared as follows. The vector pVD5 (see Example 1) was used as a template for PCR amplification using the mutagenic primer OWB41 and the M13 reverse primer (5'AGGAAACAGCTATGACCATG). Figure 5 illustrates PCR amplification using the mutagenic primer OWB41 and the M13 reverse primer. The resulting PCR product was digested with HindIII and Bam HI and subcloned into the corresponding sites of pVD4 (see Example 1). The resulting vector was digested with SalI and Bam HI and subcloned into SalI-BglII digested yeast transformation vector pTG3828 (see Example 1).

The vectors for the production of Rs-AFP2 isoforms other than yRs-AFP2/Q5M were constructed as follows. The vector pVD5 was used as a template for introducing mutations by the two-step PCR protocol of Merino et al (1992, BioTechniques, 12:508-510), with the PCR mutagenic primers being designed according to standard molecular biology techniques. For example, for Series A and B isoforms, a first PCR reaction was performed using a mutagenic primer (either OWB42, OWB43, OWB44, OWB45, OWB77, OWB47, OWB48,OWB49 or OWB50: see Figure 5) and the primer OWB35 (5'GGAATAGCCGATGGAGATCTAGGAAAACAGCTA TGACCATG, nucleotides corresponding to the M13 reverse primer underlined). The resulting PCR product was used in a second PCR reaction as a megaprimer and after 5 amplification cycles the primers OWB61 (see Example 1) and OWB36 (GGAATACCCGATCGAGATCTAGGA, corresponding to the first 24 nucleotides of OWB 35) were added. The PCR product of the second PCR reaction was subcloned in pVD4 and subsequently in pTG3828 as described above. Nucleotide sequences of all subcloned PCR products were verified by nucleotide sequencing. The obtained derivatives of pTG3828 were transformed into yeast and the RsAFP2

isoforms produced by the transformed yeast strains was purified by reversed-phase chromatography (RPC) as described in Example 2. All Rs-AFP2 isoforms had the same electrophoretic mobility as plant-derivable wild-type Rs-AFP2.

An Rs-AFP2 isoform having a mutation at both position 9 (glycine to arginine) and at position 39 (valine to arginine) may be readily made in yeast using either the G9R construct or the V39R construct as the initial PCR template instead of pVD5. The appropriate mutagenic primer is used for the second amino acid change.

EXAMPLE 4

Antifungal activity of the Rs-AFP2 isoforms

In order to assess the effect of single amino acid substitutions or deletions on the antifungal activity of Rs-AFP2, yeast-expressed and RPC-purified Rs-AFP2 isoforms (see figure 4) were tested for their specific antifungal activity. The RPC-purified Rs-AFP2 isoforms were first analysed by SDS-PAGE and the purity of the preparations was estimated to be at least 50%.

For each isoform, two independent purifications were carried out and the antifungal activity was determined in duplicate using F culmorum as a test fungus in two different media: a low ionic strength medium called SMF- (Terras et al, 1992, J Biol Chem, 267:15301-15309) and the same medium supplemented with 1mM $CaCl_2$ and 50 mM KCl called SMF+. The presence of salts in the test medium, especially salts with divalent cations, reduces the specific activity of Rs-AFP2. Seed-purified as well as yeast-expressed wild type Rs-AFP2 served as a control in the assays.

Results of preliminary tests are given in Table 1 which shows the relative specific antifungal activity against F culmorum of yeast-expressed wild-type Rs-AFP2 (yRs-AFP2) and the mutant yRs-AFP2 isoforms. The relative specific activity is expressed as the specific activity of the mutant divided by the specific activity of yRs-AFP2 and multiplied by 100. The specific activity is expressed as the reciprocal of the IC_{50} value determined on F culmorum after 48 hour of incubation in the presence of the proteins. The specific activity was measured in medium SMF- and SMF+.

TABLE 1

PROTEIN	RELATIVE SPECIFIC ACTIVITY (%) IN MEDIUM	
	SMF-	SMF+
yRs-AFP2	100	100
SERIES A		
yRs-AFP2/Q5M	100	100
yRs-AFP2/T10G	30	<16
yRs-AFP2/G16M	151	114
yRs-AFP2/A31W	15	<5
yRs-AFP2/Y38G	30	<4
yRs-AFP2/F40M	30	23
yRs-AFP2/K44Q	100	114
yRs-AFP2/Y48I	38	114
SERIES B		
yRs-AFP2/P7-	8	17
yRs-AFP2/P41-	4	<10
SERIES C		
yRs-AFP2/P7R	33	84
yRs-AFP2/G9R	116	285
yRs-AFP2/S12R	67	31
yRs-AFP2/I26R	76	82
yRs-AFP2/L28R	39	-
yRs-AFP2/N37R	100	80
yRs-AFP2/V39R	74	114
yRs-AFP2/A42R	44	26
yRs-AFP2/I46R	22	-
yRs-AFP2/F49R	18	22

It is seen that certain mutations cause a major decrease in antifungal activity while certain proteins (notably Q5M and V39R) maintain their antifungal activity. However, two mutations cause an increase in antifungal activity. The isoform with

the G16M mutation shows an increased activity in low salt (SMF-), although activity is not so significantly different in high salt (SMF+). The mutant G9R is approximately three times more active than yRs-AFP2 in high salt (SMF+), although activity is not so significantly different in low salt (SMF-).

Table 2 shows results from further comparative tests of the Rs-AFP2 isoforms in which experiments were carried out in triplicate. IC_{50} values were measured after 72 hours growth in low salt (SMF-) and high salt (SMF+) media. Deviations are given as standard error of the mean (sem) based on the triplicate experiments. In SMF-, the medium without added salts, most of the derivatives show no decrease or only a minor decrease in antifungal activity, while in SMF+, the medium with added salts, there is a significant decrease in antifungal activity for several Rs-AFP2 isoforms. Substitutions that apparently have little effect on the antifungal activity in SMF- (low salt medium) include G9R, V39R, Q5M and G16M. However, in SMF+ (high salt medium) these four isoforms (in particular G39R and V39R) show an increased antifungal activity.

TABLE 2

PROTEIN	SMF-		SMF+	
	IC_{50}	sem	IC_{50}	sem
yRs-AFP2	2.7	0.6	8.5	2.7
yRs-AFP2/Q5M	4.1	0.2	5.4	1.2
yRs-AFP2/T10G	11.0	4.2	>100	
yRs-AFP2/W11S	16.0	5.7	>100	
yRs-AFP2/G16M	2.2	0.3	5.0	0.9
yRs-AFP2/A31W	30.0	5.0	>100	
yRs-AFP2/H33A	32.0	8.7	>100	
yRs-AFP2/Y38G	42.0	17.0	>200	
yRs-AFP2/F40M	16.0	6.7	54.0	13.0
yRs-AFP2/P41-	100.0	15.0	>200	
yRs-AFP2/K44Q	3.6	0.4	36.0	9
yRs-AFP2/Y48I	9.3	1.0	11.0	2.0
yRs-AFP2/P7R	6.8	2.4	8.8	1.0

yRs-AFP2/G9R	3.0	0.5	3.3	0.6
yRs-AFP2/S12R	3.5	1.0	20.0	6.0
yRs-AFP2/I26R	7.2	0.8	9.6	3.7
yRs-AFP2/L28R	6.4	1.4	>100	
yRs-AFP2/N37R	2.8	0.3	7.0	1.8
yRs-AFP2/V39R	4.0	0.2	3.2	0.3
yRs-AFP2/A42R	4.2	2.5	18.0	5.2
yRs-AFP2/I46R	12.0	2.4	>40	
yRs-AFP2/F49R	22.0	4.8	23.0	3.0

Figure 6 is a graph of relative specific antifungal activity of the Rs-AFP isoforms as determined on F culmorum in medium SMF+. The specific antifungal activity ($1/IC_{50}$) of Rs-AFP2 was set at 100. Bars without indication of standard deviation represent maximum values; actual values may be even lower. The G9R and V39R isoforms are particularly active, with the Q5M and G16M isoforms also showing enhanced activity.

EXAMPLE 5

Rs-AFP2/G9R and Rs-AFP2/V39R isoforms: further tests of antifungal activity

Isoforms Rs-AFP2/G9R and Rs-AFP2/V39R were subjected to further tests as their antifungal activity in SMF+, in contrast with that of other isoforms, was approximately two-fold higher than that of wild-type Rs-AFP2. Their antifungal activity was determined in SMF with increasing Ca^{2+} or K^+ concentration and compared with that of plant-derivable wild type Rs-AFP2 (isolated from seed) as well as yeast purified Rs-AFP2. Figure 7 is a graph of the percentage growth inhibition of F culmorum caused by 10 µg/ml of yeast-purified Rs-AFP2 (open circles), seed-purified Rs-AFP2 (closed circles), Rs-AFP2/G9R (squares) and Rs-AFP2/V39R (triangles) in a medium consisting of SMF with varying concentrations of $CaCl_2$ (panel A) and KCl (panel B).

As shown in Figure 7, the antifungal activity of yRS-AFP2/G9R and yRS-AFP2/V39R was less reduced by the presence of cations in the growth medium than the activity of wild-type Rs-AFP2. At a concentration of 10 µg/ml, both G9R

and V39R caused complete inhibition of the growth of F culmorum in the presence of 5mM $CaCl_2$ whereas wild type Rs-AFP2 was basically inactive under the same conditions. At 10 µg/ml, wild-type Rs-AFP2 was fully active against F culmorum only when the $CaCl_2$ concentration was equal to or lower than 1.25 mM. Similarly, the activity of wild type Rs-AFP2 was drastically reduced in the presence of 100 mM KCl. whereas Rs-AFP2 isoforms G9R and V39R were still fully inhibitory to fungal growth.

Thus the G9R and V39R Rs-AFP2 isoforms show no increased activity in the low ionic strength medium, but their activity is more resistant to the presence of cations in comparison with wild-type Rs-AFP2. As relatively high ionic strength conditions occur in all cell compartments, such Rs-AFP2 isoforms displaying a decreased cation antagonism may be useful for plant transformation to obtain disease resistant crops. An Rs-AFP2 isoform yRs-AFP2/G9R/V39R having a mutation at both position 9 (glycine to arginine) and at position 39 (valine to arginine) would carry a net +2 positive charge compared to Rs-AFP2 and is expected to show antifungal activity having an increased salt-tolerence above that even of the individual isoforms G9R or V39R.

The antifungal activity of the G9R and V39R Rs-AFP2 isoforms was also assessed on a set of seven different phytopathogenic fungi in three media differing in ionic strength: SMF-, SMF plus 1mM $CaCl_2$ and 50mM KCl, and SMF plus 5mM $CaCl_2$ and 50mM KCl. The fungi tested were: Alternaria brassicicola, Ascochyta pisi, Botrytis cinerea, Fusarium culmorum, Nectria haematococca, Phoma betae and Verticillium dahliae. The results are shown in Table 3. All IC_{50} values were recorded after 72 hours of growth except for IC_{50} values on V dahliae and F culmorum which were determined after 96 hours of growth.

The data in Table 3 show that the relative strength of the Rs-AFP2 isoforms may be dependent on the test organism. The activity of the G9R and V39R isoforms against A brassicola, A pisi and B cinerea was comparable to the activity of Rs-AFP2, while Rs-AFP2 appeared to be more active against P betae. However, on three fungi (F culmorum, N haematococca and V dahliae) the isoforms Rs-AFP2/G9R and Rs-AFP2/V39R were more active than Rs-AFP2 itself particularly in the SMF media with added salts. For example, in the medium SMF

plus 1mM $CaCl_2$ and 50mM KCl, Rs-AFP2/G9R was approximately three-fold more active than Rs-AFP2 against these three fungi while Rs-AFP2/V39R was approximately two-fold more active than Rs-AFP2 against F culmorum and five-fold more active against N haematococca and V dahliae. The three fungi against which Rs-AFP2/V39R and Rs-AFP2/G9R are more active than Rs-AFP2 belong to the same family of fungi, Namely the Nectriaceae.

TABLE 3

FUNGUS	Rs-AFP2	SMF G9R	V39R	IC_{50} VALUES (µG/ml) SMF+1 mM $CaCl_2$/50mM Kcl			SMF+5mM $CaCl_2$/50mM Kcl		
				Rs-AFP2	G9R	V39R	Rs-AFP2	G9R	V39F
A BRASSIC-CIOLA	3.2	2.6	2.5	>50	>50	50	>100	>100	>100
A PISI	1.9	1.6	2.0	>50	>50	>50	>100	>100	>100
B CINEREA	1.8	1.9	1.6	>50	>50	>50	>100	>100	>100
F CULMORUM	2.1	2.2	2.2	4.6	1.5	2.3	22.0	7.2	7.0
N HAEMA-TOCOCCA	2.0	2.0	2.1	48.0	16.0	9.0	>100	100	62.0
P BETAE	0.9	2.0	1.4	14.0	2.50	40.0	27.0	>100	70.0
V DAHLIAE	1.0	0.5	0.4	11.0	4.0	2.3	50.0	17.0	6.0

CLAIMS

1. An antifungal protein having an amino acid sequence which is substantially homologous to the Rs-AFP2 sequence shown in Figure 1 and containing at least one mutation selected from the group a basic residue at position 9, a basic residue at position 39, a hydrophobic residue at position 5 and a hydrophobic residue at position 16.

2. An antifungal protein according to claim 1 and containing at least one mutation selected from the group consisting of an arginine residue at position 9, an arginine residue at position 39, a methionine residue at position 5 and a methionine residue at position 16.

3. An antifungal protein according to claim 2 wherein said protein has a mutation to arginine at position 9 and a mutation to arginine at position 39.

4. An antifungal peptide comprising at least six amino acid residues identical to a run of amino acid residues in an antifungal protein according to claim 1 said run of residues including at least one of the mutated residues.

5. A DNA sequence encoding an antifungal protein as claimed in any of Claims 1 to 4.

6. A vector containing a DNA sequence as claimed in claim 5.

7. A biological system including DNA as claimed in claim 5 such that the encoded protein is expressed.

8. A biological system as claimed in claim 7 which is a plant.

9. A plant having improved resistance to a fungal pathogen and containing recombinant DNA as claimed in Claim 5.

10. An antifungal composition comprising a protein or peptide as claimed in any of Claims 1 to 4.

11. A process of combating fungi or bacteria which comprises exposing them to the proteins as claimed in any of Claims 1 to 4 or a composition as claimed in Claim 10.

Fig.1.

```
                1           11          21          31          41          51
Rs-AFP1     QKLCERPSGT WSGVCGNNNA CKNQCINLEK ARHGSCNYVF PAHKCICYFP C
Rs-AFP2     QKLCQRPSGT WSGVCGNNNA CKNQCIRLEK ARHGSCNYVF PAHKCICYFP C
Rs-AFP3     -KLCERSSGT WSGVCGNNNA CKNQCIRLEG AQHGSCNYVF PAHKCICYFP C
Rs-AFP4     QKLCERSSGT WSGVCGNNNA CKNQCINLEG ARHGSCNYIF PYHRCICYFP C
Br-AFP1     QKLCERPSGT WSGVCGNNNA CKNQCIN
Br-AFP2     QKLCERPSGT ?SGVCGNNNA CKNQCIR
Bn-AFP1     QKLCERPSGT WSGVCGNNNA CKNQCINLEK
Bn-AFP2     QKLCERPSGT WSGVCGNNNA CKN
Sa-AFP1     QKLCERPSGT WSGVCGNNNA CKNQC
Sa-AFP2     QKLCQRPSGT WSGVCGNNNA CRNQCI
At-AFP1     QKLCERPSGT WSGVCGNSNA CKNQCIN
```

Fig.2.

```
GTTTTATTAGTGATCATGGCTAAGTTTGCGTCCATCATCGCACTT      45
                M  A  K  F  A  S  I  I  A  L

CTTTTTGCTGCTCTTGTTCTTTTTTGCTGCTTTCGAAGCACCAACA    90
 L  F  A  A  L  V  L  F  A  A  F  E  A  E  T

ATGGTGGAAGCACAGAAGTTGTGCGAAAGGCCAAGTGGGACATGG    135
 M  V  E  A [Q  K  L  C  E  R  P  S  G  T  W]

TCAGGAGTCTGTGGAAACAATAACGCATGCAAGAATCAGTGCATT     180
[S  G  V  C  G  N  N  N  A  C  K  N  Q  C  I]

AACCTTGAGAAAGCACGACATGGATCTTGCAACTATGTCTTCCCA     225
[N  L  E  K  A  R  H  G  S  C  N  Y  V  F  P]

GCTCACAAGTGTATCTGCTACTTTCCTTGTIAATTTATCGCAAAC     270
[A  H  K  C  I  C  Y  F  P  C]  *

TCTTTGGTGAATAGTTTTTATGTAATTTACACAAAATAAGTCAGT     315

GTCACTATCCATGAGTGATTTTAAGACATGTACCAGATATGTTAT     360

GTTGGTTCGGTTATACAAATAAAGTTTTATTCACCAAAAAAAAAA     405

AAAAAAAAA                                         414
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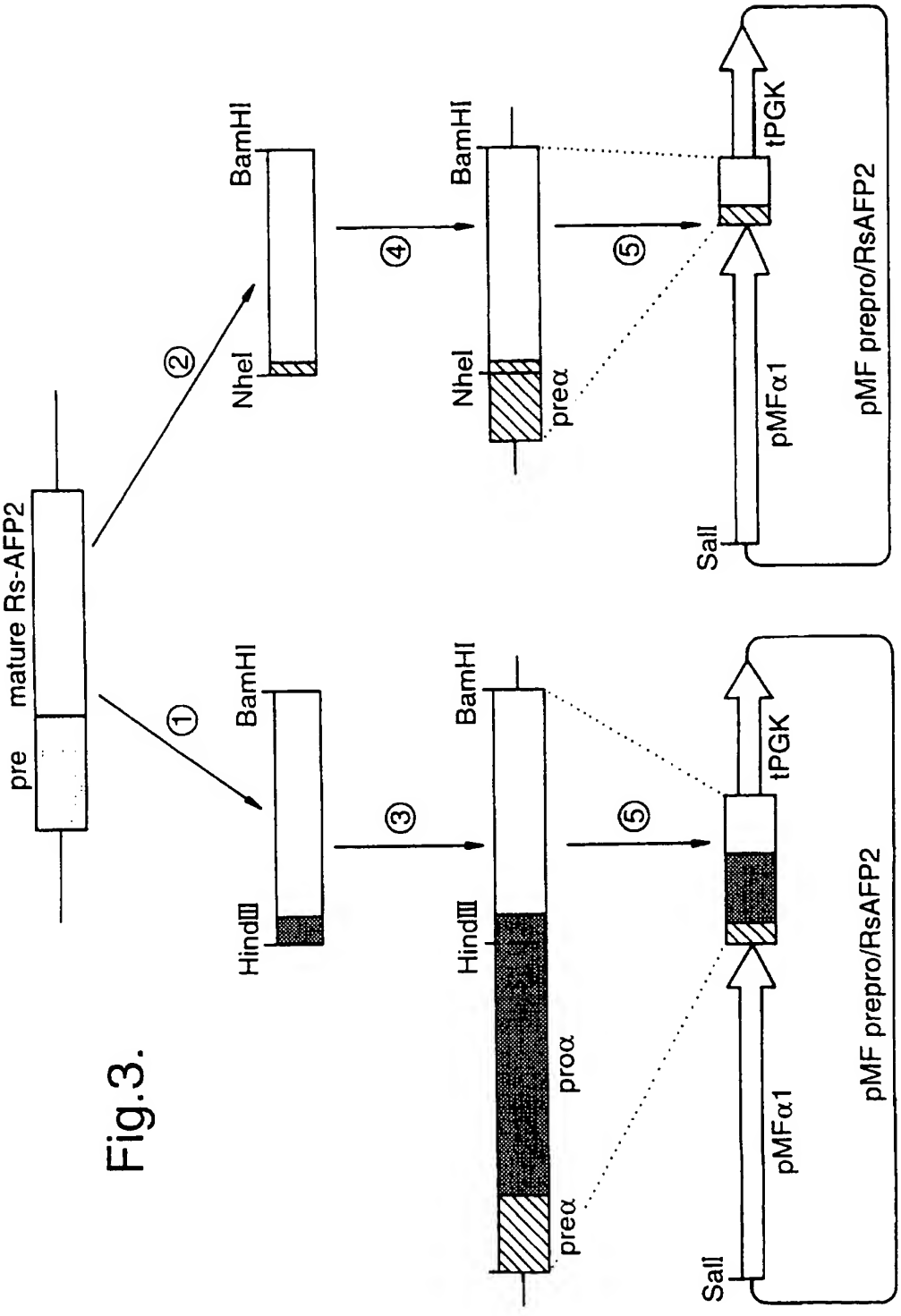

pre
mature Rs-AFP2
①
②
③
④
⑤
BamHI
HindIII
NheI
proα
preα
tPGK
pMFα1
SalI
pMF prepro/RsAFP2

Fig.3.

Fig.4.

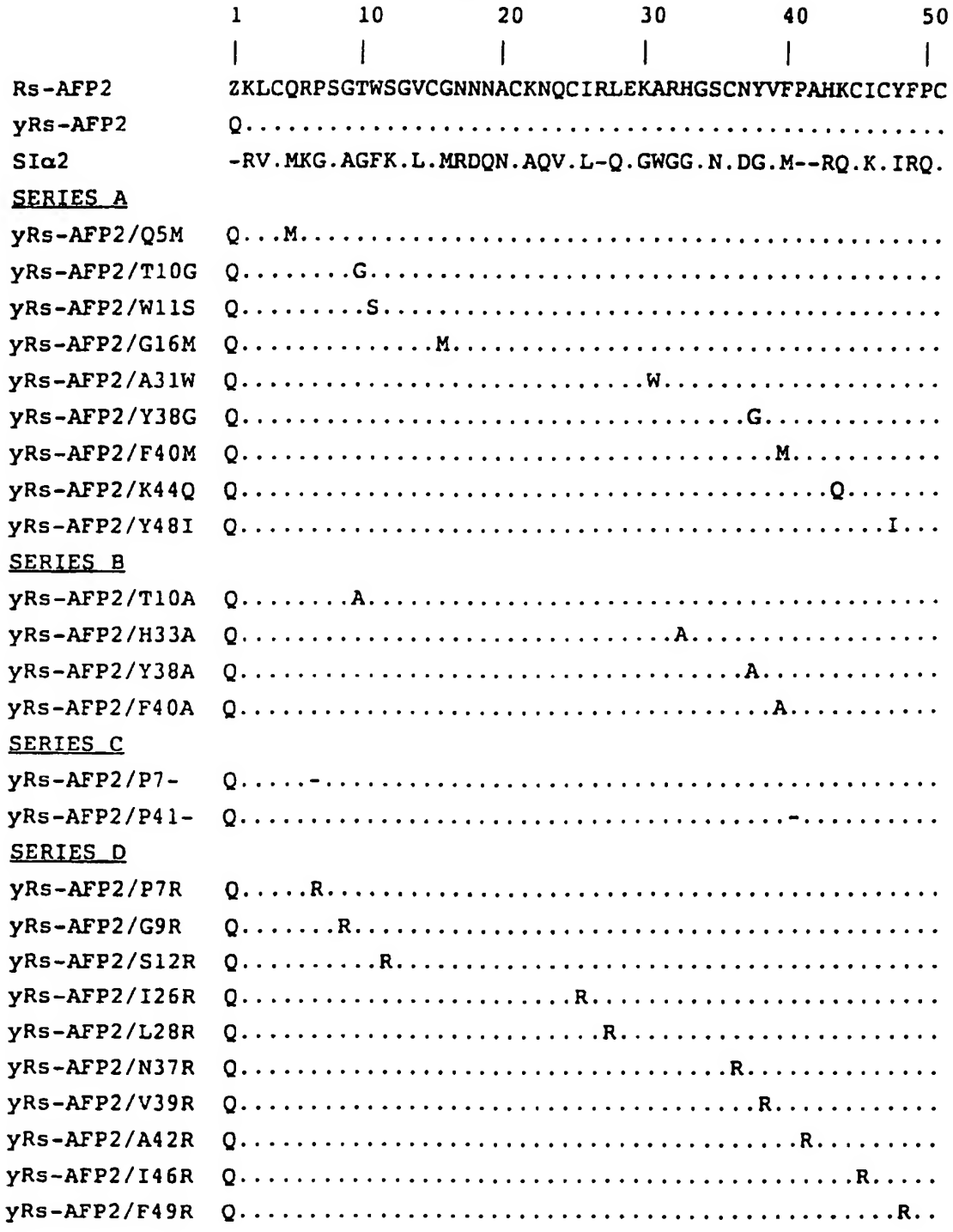

1 10 20 30 40 50
Rs-AFP2 ZKLCQRPSGTWSGVCGNNNACKNQCIRLEKARHGSCNYVFPAHKCICYFPC
yRs-AFP2 Q
SIα2 -RV.MKG.AGFK.L.MRDQN.AQV.L-Q.GWGG.N.DG.M--RQ.K.IRQ.
SERIES A
yRs-AFP2/Q5M Q...M
yRs-AFP2/T10G Q.....G
yRs-AFP2/W11S Q.....S
yRs-AFP2/G16M Q.....M
yRs-AFP2/A31W Q.....W
yRs-AFP2/Y38G Q.....G
yRs-AFP2/F40M Q.....M
yRs-AFP2/K44Q Q.....Q
yRs-AFP2/Y48I Q.....I
SERIES B
yRs-AFP2/T10A Q.....A
yRs-AFP2/H33A Q.....A
yRs-AFP2/Y38A Q.....A
yRs-AFP2/F40A Q.....A
SERIES C
yRs-AFP2/P7- Q.....-
yRs-AFP2/P41- Q.....-
SERIES D
yRs-AFP2/P7R Q.....R
yRs-AFP2/G9R Q.....R
yRs-AFP2/S12R Q.....R
yRs-AFP2/I26R Q.....R
yRs-AFP2/L28R Q.....R
yRs-AFP2/N37R Q.....R
yRs-AFP2/V39R Q.....R
yRs-AFP2/A42R Q.....R
yRs-AFP2/I46R Q.....R
yRs-AFP2/F49R Q.....R

Fig.5.

	^{1}Q	^{2}K	^{3}L	^{4}C	^{5}Q	^{6}R	^{7}P					
	CAG	AAG	TTG	TGC	CAA	AGG	CCA	AG				
OWB41:AATAAGCTTTGGACAAGAGA	CAG	AAG	TTG	TGC	ATG	AGG	CCA	AG				
	Q	K	L	C	M	R	P					
		^{3}L	^{4}C	^{5}Q	^{6}R	^{7}P	^{8}S	^{9}G	^{10}T	^{11}W		
		TTG	TGC	CAA	AGG	CCA	AGT	GGG	ACA	TGG		
OWB42:TTG		TTG	TGC	CAA	AGG	---	AGT	GGG	ACA	TGG		
		L	C	Q	R	-	S	G	T	W		
			^{7}P	^{8}S	^{9}G	^{10}T	^{11}W	^{12}S				
			CCA	AGT	GGG	ACA	TGG	TCA	GG			
OWB43:CCA			CCA	AGT	GGG	GGT	TGG	TCA	GG			
			P	S	G	G	W	S				
				^{8}S	^{9}G	^{10}T	^{11}W	^{12}S	^{13}G	^{14}V		
				AGT	GGG	ACA	TGG	TCA	GGA	GTC		
OWB44:AGT				AGT	GGG	ACA	TCC	TCA	GGA	GTC		
				S	G	T	S	S	G	V		
					^{13}G	^{14}V	^{15}C	^{16}G	^{17}N	^{18}N	^{19}N	
					GGA	GTC	TGT	GGA	AAC	AAT	AAC	GC
OWB45:GGA					GGA	GTC	TGT	ATG	AAC	AAT	AAC	GC
					G	V	C	M	N	N	N	

Fig.5 (Cont).

	^{35}S	^{36}C	^{37}Q	^{38}Y	^{39}V	^{40}F		^{42}A		^{43}H	^{44}K		
	TCT	TGC	AAC	TAT	GTC	TTC	CC						
OWB77:	TCT	TGC	AAC	GGT	GTC	TTC	CC						
	S	C	Q	G	V	F							
	^{36}C	^{37}Q	^{38}Y	^{39}V	^{40}F	^{41}P		^{42}A		^{43}H	^{44}K		
	TGC	AAC	TAT	GTC	TTC	CCA		GCT	C				
OWB47:	TGC	AAC	TAT	GTC	ATG	CCA		GCT	C				
	C	Q	Y	V	M	P		A		H	K		
	^{37}Q	^{38}Y	^{39}V	^{40}F	^{41}P	^{42}A		^{43}H		^{44}K			
	AAC	TAT	GTC	TTC	CCA	GCT		CAC		CAC	AAG	TG	
OWB48:	AAC	TAT	GTC	TTC	---	GCT		CAC		CAC	AAG	TG	
	Q	Y	V	F	-	A		H		H	K		
	^{40}F	^{41}P	^{42}A	^{43}H	^{44}K	^{45}C		^{46}I					
	TTC	CCA	GCT	CAC	AAG	TGT		ATC		TG			
OWB49:	TTC	CCA	GCT	CAC	CAA	TGT		ATC		TG			
	F	P	A	H	Q	C		I					
	^{45}C	^{46}I	^{47}C	^{48}Y	^{49}F	^{50}C							
	TGT	ATC	TGC	TAC	TTT	CCT		TG					
OWB50:	TGT	ATC	TGC	ATC	TTT	CCT		TG					
	C	I	C	I	F	C							

Fig.6.

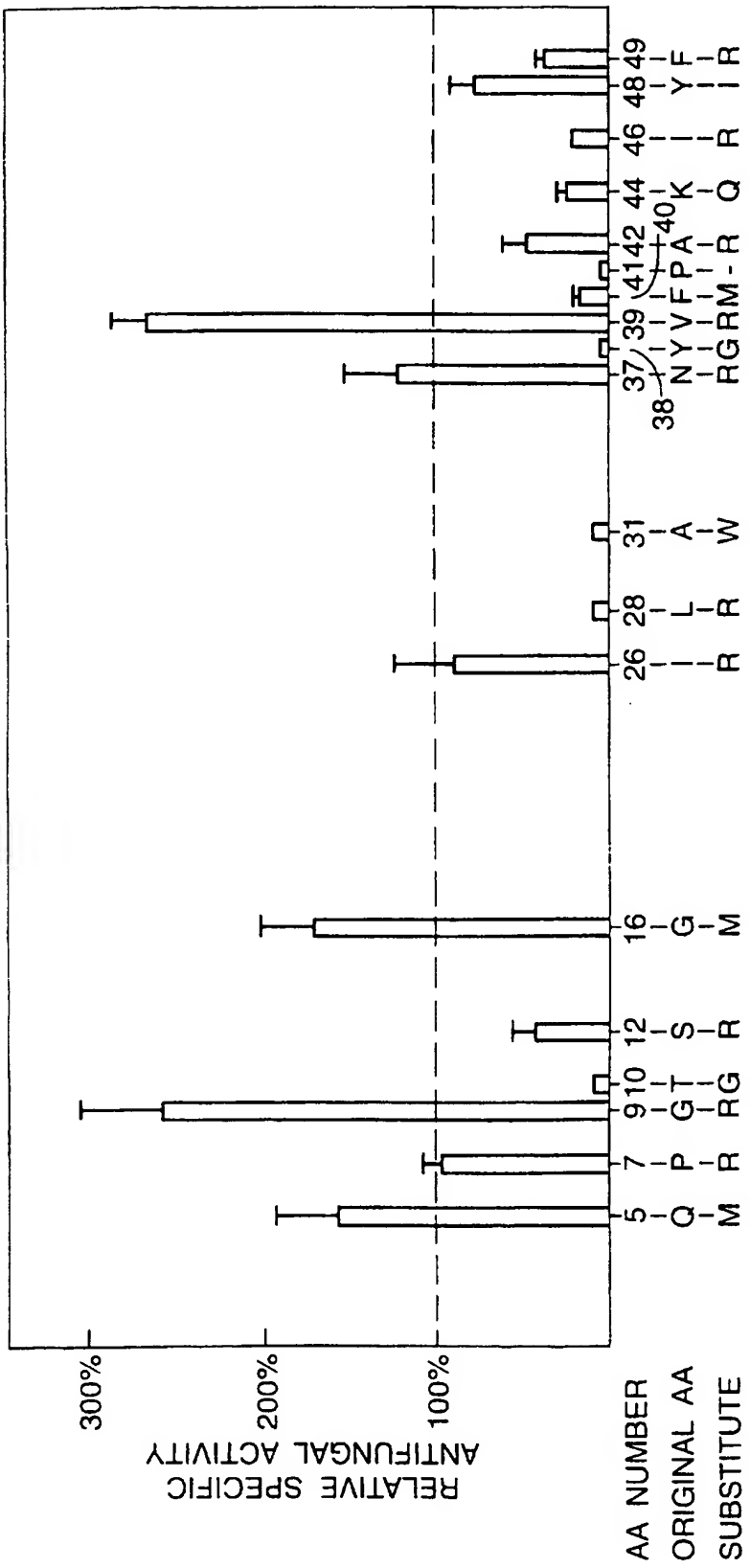
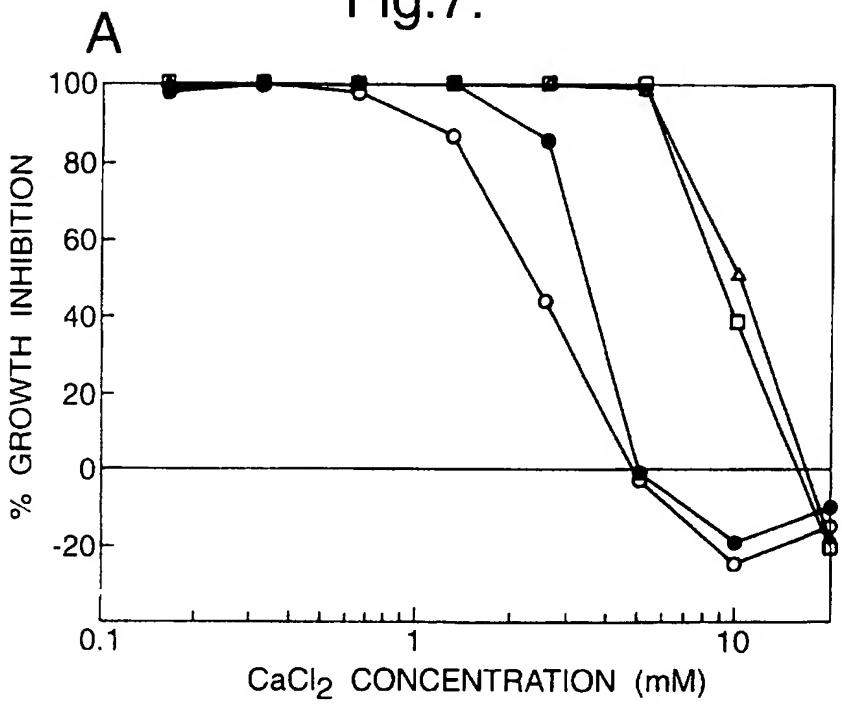
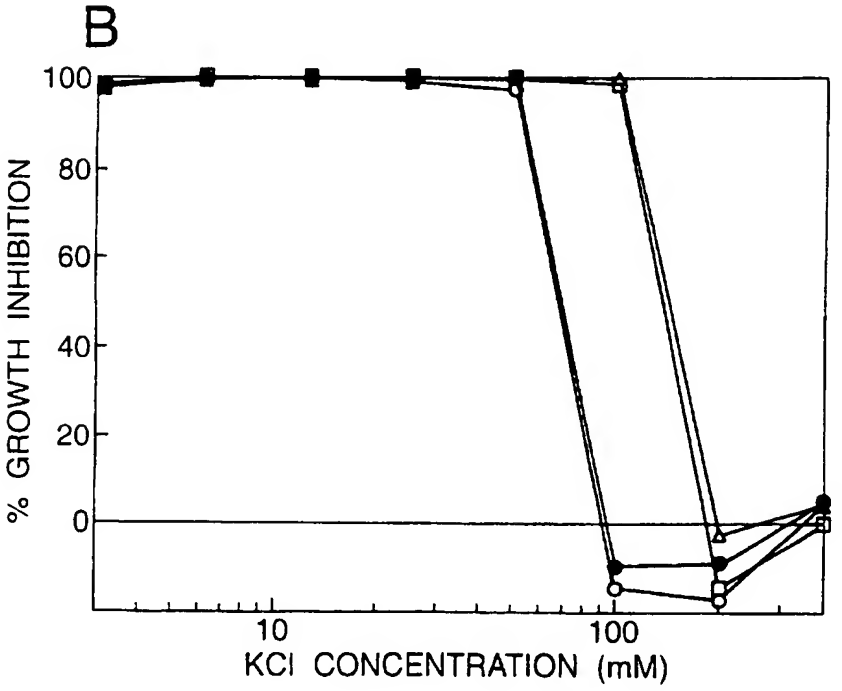

RELATIVE SPECIFIC ANTIFUNGAL ACTIVITY
300%
200%
100%
AA NUMBER
ORIGINAL AA
SUBSTITUTE
5 Q M
7 P R
9 G R
10 T G
12 S R
16 G M
26 R R
28 L R
31 A W
37 N R
38 Y G
39 V R
40 F M
41 P -
42 A R
44 K Q
46 R R
48 Y I
49 F R

Fig.7.


A
100
80
60
40
20
0
-20
% GROWTH INHIBITION
0.1
1
10
CaCl2 CONCENTRATION (mM)


B
100
80
60
40
20
0
% GROWTH INHIBITION
10
100
KCl CONCENTRATION (mM)